JOURNAL OF BACTERIOLOGY, Oct. 2010, p. 5071–5080 Vol. 192, No. 19
0021-9193/10/$12.00 doi:10.1128/JB.00575-10

Characterization of the PduS Cobalamin Reductase of *Salmonella enterica* and Its Role in the Pdu Microcompartment▽†

Shouqiang Cheng and Thomas A. Bobik*

Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa 50011

Received 18 May 2010/Accepted 14 July 2010

Salmonella enterica **degrades 1,2-propanediol (1,2-PD) in a coenzyme B_{12} (adenosylcobalamin, AdoCbl)-dependent fashion. *Salmonella* obtains AdoCbl by assimilation of complex precursors, such as vitamin B_{12} and hydroxocobalamin. Assimilation of these compounds requires reduction of their central cobalt atom from Co^{3+} to Co^{2+} to Co^{+}, followed by adenosylation to AdoCbl. In this work, the His_6-tagged PduS cobalamin reductase from *S. enterica* was produced at high levels in *Escherichia coli*, purified, and characterized. The anaerobically purified enzyme reduced cob(III)alamin to cob(II)alamin at a rate of 42.3 ± 3.2 μmol min^{-1} mg^{-1}, and it reduced cob(II)alamin to cob(I)alamin at a rate of 54.5 ± 4.2 nmol min^{-1} mg^{-1} protein. The apparent K_m values of PduS-His_6 were 10.1 ± 0.7 μM for NADH and 67.5 ± 8.2 μM for hydroxocobalamin in cob(III)alamin reduction. The apparent K_m values for cob(II)alamin reduction were 27.5 ± 2.4 μM with NADH as the substrate and 72.4 ± 9.5 μM with cob(II)alamin as the substrate. High-performance liquid chromatography (HPLC) and mass spectrometry (MS) indicated that each monomer of PduS contained one molecule of noncovalently bound flavin mononucleotide (FMN). Genetic studies showed that a *pduS* deletion decreased the growth rate of *Salmonella* on 1,2-PD, supporting a role in cobalamin reduction *in vivo*. Further studies demonstrated that the PduS protein is a component of the Pdu microcompartments (MCPs) used for 1,2-PD degradation and that it interacts with the PduO adenosyltransferase, which catalyzes the terminal step of AdoCbl synthesis. These studies further characterize PduS, an unusual MCP-associated cobalamin reductase, and, in conjunction with prior results, indicate that the Pdu MCP encapsulates a complete cobalamin assimilation system.**

Coenzyme B_{12} (adenosylcobalamin, AdoCbl) is an indispensable cofactor for a variety of enzymes that are widely distributed among microbes and higher animals (2, 55). Organisms obtain AdoCbl by *de novo* synthesis or by assimilation of complex precursors, such as vitamin B_{12} (cyanocobalamin, CN-Cbl) and hydroxocobalamin (OH-Cbl), which can be enzymatically converted to AdoCbl. *De novo* synthesis occurs only in prokaryotes, but the assimilation of complex precursors is more widespread, taking place in many microbes and in higher animals (56). A model for the assimilation of CN-Cbl and OH-Cbl to AdoCbl, based on work done in a number of laboratories, is shown in Fig. 1. CN-Cbl is first reductively decyanated to cob(II)alamin (22, 30, 68). Next, cob(II)alamin is reduced to cob(I)alamin, and ATP:cob(I)alamin adenosyltransferase (ATR) transfers a 5′ deoxyadenosyl group from ATP to cob(I)alamin to form AdoCbl (10, 11, 28, 29, 35, 63, 64, 72). Studies indicate that prior to reduction cob(II)alamin binds the ATR and undergoes a transition to the 4-coordinate base-off conformer (41, 48, 59, 61, 62). Transition to the 4-coordinate state raises the midpoint potential of the cob(II)alamin/cob(I)alamin couple by about 250 mV, facilitating reduction (60). OH-Cbl assimilation occurs by a similar pathway except that the first step is reduction of OH-Cbl to cob(II)alamin by cobalamin reductase or by the reducing environment of the cell (19, 69).

The pathway used for the assimilation of OH-Cbl and CN-Cbl is also used for intracellular cobalamin recycling. During catalysis the adenosyl group of AdoCbl is periodically lost due to by-reactions and is usually replaced by a hydroxyl group, resulting in the formation of an inactive OH-Cbl enzyme complex (66). Cobalamin recycling begins with a reactivase that converts the inactive OH-Cbl–enzyme complex to OH-Cbl and apoenzyme (43, 44). Next, the process described in Fig. 1 converts OH-Cbl to AdoCbl, which spontaneously associates with apoenzyme to form active holoenzyme (43, 44, 66). In the organisms that have been studied, cobalamin recycling is essential, and genetic defects in this process block AdoCbl-dependent metabolism (3, 16, 29).

Salmonella enterica degrades 1,2-propanediol (1,2-PD) via an AdoCbl-dependent pathway (27). 1,2-PD is a major product of the anaerobic degradation of common plant sugars rhamnose and fucose and is thought to be an important carbon and energy source in natural environments (38, 46). Twenty-four genes for 1,2-PD utilization (*pdu*) are found in a contiguous cluster (*pocR*, *pduF*, and *pduABB′CDEGHJKLMNOPQSTUVWX*) (7, 27). This locus encodes enzymes for the degradation of 1,2-PD and cobalamin recycling, as well as proteins for the formation of a bacterial microcompartment (MCP) (7). Bacterial MCPs are simple proteinaceous organelles used by diverse bacteria to optimize metabolic pathways that have toxic or volatile intermediates (6, 13, 14, 71). They are polyhedral in shape, 100 to 150 nm in cross section (about the size of a large virus), and consist of a protein shell that encapsulates sequentially acting metabolic enzymes. Sequence analyses indicate

* Corresponding author. Mailing address: Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011. Phone: (515) 294-4165. Fax: (515) 294-0453. E-mail: bobik@iastate.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 23 July 2010.

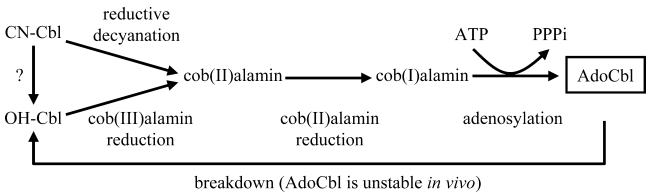

FIG. 1. Cobalamin assimilation and recycling pathway. Many organisms are able to take up CN-Cbl and OH-Cbl and convert them to the active coenzyme form, AdoCbl. This process involves reduction of the central cobalt atom of the corrin ring followed by addition of a 5′ deoxyadenosyl (Ado) group via a carbon-cobalt bond. The Ado group is unstable *in vivo*, and AdoCbl breaks down to form OH-Cbl. Consequently, cobalamin recycling is required for AdoCbl-dependent processes, and recycling uses the same pathway that functions in the assimilation of cobalamin from the environment. PPPi, triphosphate.

that MCPs are produced by 20 to 25% of all bacteria and function in seven or more different metabolic processes (14). The function of the Pdu MCP is to confine the propionaldehyde formed in the first step of 1,2-PD degradation in order to mitigate its toxicity and prevent DNA damage (7, 23, 24, 51). Prior studies indicate that 1,2-PD traverses the protein shell and enters the lumen of the Pdu MCP, where it is converted to propionaldehyde and then to propionyl-coenzyme A (CoA) by AdoCbl-dependent diol dehydratase (DDH; PduCDE) and propionaldehyde dehydrogenase (PduP) (8, 33). Propionyl-CoA then exits the MCP into the cytoplasm, where it is converted to 1-propanol or propionate or enters central metabolism via the methylcitrate pathway (25, 47). The shell of the Pdu MCP is thought to limit the diffusion of propionaldehyde in order to protect cytoplasmic components from toxicity. The Pdu MCP was purified, and 14 major polypeptide components were identified (PduABB′CDEGHJKOPTU), all of which are encoded by the *pdu* locus (23). PduABB′JKTU are confirmed or putative shell proteins (23, 24, 51). PduCDE and PduP catalyze the first 2 steps of 1,2-PD degradation as described above (7, 8, 23, 33). The PduO and PduGH enzymes are used for cobalamin recycling. PduO is an adenosyltransferase (29), and PduGH is a homolog of the *Klebsiella* DDH reactivase, which mediates the removal of OH-Cbl from an inactive OH-Cbl–DDH complex (43, 44). However, a reductase which is also required for cobalamin recycling was not previously identified as a component of the Pdu MCP (23). This raises the question of how cobalamin is recycled for the AdoCbl-dependent DDH that resides within the Pdu MCP.

Prior studies indicated that the PduS enzyme (which is encoded by the *pdu* locus) is a cobalamin reductase (52). Very recently PduS was purified from *S. enterica* and shown to be a flavoprotein that can mediate the reduction of 4-coordinate cob(II)alamin bound to ATR but was not further characterized (40). In this study, PduS from *S. enterica* is purified and more extensively characterized, including identification of its cofactor requirements and kinetic properties. In addition, we show that PduS is a component of the Pdu MCP. This finding in conjunction with prior work indicates that, in addition to 1,2-PD degradative enzymes, the Pdu MCP encapsulates a complete cobalamin recycling system.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The rich media used were Luria-Bertani/Lennox (LB) medium (Difco, Detroit, MI) (42) and Terrific Broth (TB) (MP Biomedicals, Solon, OH) (65). The minimal medium used was no-carbon E (NCE) medium (4, 67).

Chemicals and reagents. Antibiotics, OH-Cbl, CN-Cbl, AdoCbl, iodoacetate, DNase I, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and corynebacterial sarcosine oxidase were from Sigma Chemical Company (St. Louis, MO). IPTG (isopropyl-β-*d*-thiogalactopyranoside) was from Diagnostic Chemicals Limited (Charlotteville, PEI, Canada). *PfuUltra* high-fidelity DNA polymerase was from Stratagene (La Jolla, CA). *Taq* DNA polymerase, restriction enzymes, and T4 DNA ligase were from New England Biolabs (Beverly, MA). Other chemicals were from Fisher Scientific (Pittsburgh, PA).

Construction of plasmids and a *pduS* deletion mutant. The *pduS* gene was amplified by PCR (using genomic DNA of *S. enterica* as the template), then cloned into the pET-41a vector (Novagen, Cambridge, MA) using NdeI and HindIII restriction sites incorporated into the PCR primers as previously described (50). A gene for the production of PduS-His_6 was similarly cloned, with the sequence encoding the His_6 tag incorporated into the reverse PCR primer. The resulting plasmids, pET-41a-*pduS* and pET-41a-*pduS*-His_6, as well as pET-41a without insert were introduced into *Escherichia coli* C41(DE3) (Lucigen, Middleton, WI). The native *pduS* gene was also cloned into pLAC22, between BglII and HindIII restriction sites, for complementation experiments. The native *pduS* and *pduO* genes were cloned into both pBT and pTRG (Stratagene) between BamHI and XhoI sites for two-hybrid analyses. All of the above inserts were verified by DNA sequencing.

The *pduS* deletion mutant was constructed by a PCR-based method (15) using primers *pduS*-DKF (CCAATGCCGAAGCCATTCGGGAACTGCTGGAGGA ACTGCTATAATTGTAGGCTGGAGCTGCTTCG) and *pduS*-DKR (CTAAA ATTCCTATAGCCTGAGACATGGTTAACCTCTTACAGATATGAATATC CTCCTTAGTTC). These primers were designed to remove nearly the entire *pduS* coding sequence but leave predicted translation signals of the adjacent

TABLE 1. Bacterial strains used in this study

Species and strain	Genotype	Source
E. coli		
BE118	BL21(DE3) RIL/pTA925-*pduO*	Lab collection
C41(DE3)	F^- *ompT hsdSB*(r_B^- m_B^-) *gal dcm* (DE3)	Lucigen
BE1355	C41(DE3)/pET-41a	This work
BE1356	C41(DE3)/pET-41a-*pduS*	This work
BE1374	C41(DE3)/pET-41a-*pduS*-His_6	This work
BacterioMatch II two-hybrid system reporter strain	Δ(*mcrA*)*183* (*mcrCB-hsdSMR-mrr*)*173 endA1 hisB supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *lacI*^{q} *HIS3 aadA* Kan^r]	Stratagene
S. enterica serovar Typhimurium LT2		
BE1352	Δ*pduS*::frt	This work
BE287	LT2/pLAC22	Lab collection
BE1353	Δ*pduS*::frt/pLAC22	This work
BE1354	Δ*pduS*::frt/pLAC22-*pduS*	This work

genes (*pduQ* and *pduT*) intact. The presence of the *pduS* deletion was verified by PCR as described previously (15).

Growth of expression strains and purification of PduS-His$_6$. Two hundred milliliters of TB containing 25 mg/ml kanamycin was inoculated with 2 ml of BE1374 grown in similar medium, and the cells were cultivated in a 1-liter baffled Erlenmeyer flask at 37°C with shaking at 275 rpm. When the culture reached an optical density at 600 nm (OD_{600}) of about 0.5, riboflavin (10 μM), ferric ammonium citrate (50 μg/ml), L-cysteine (1 mM), and IPTG (0.1 mM) were added. The culture was incubated for an additional 18 h at 30°C with 200-rpm shaking. Cells were then harvested by centrifugation at 4°C and 6,000 × *g* for 10 min and used immediately for protein purification. Cells were resuspended in buffer A [50 mM potassium phosphate, pH 7.5, 300 mM NaCl, 5 mM β-mercaptoethanol, 0.4 mM AEBSF {4-(2-aminoethyl) benzenesulfonyl fluoride-HCl}] containing 20 mM imidazole and a few crystals of bovine pancreas DNase I. Cells were broken using a French pressure cell (SLM Aminco, Rochester, NY) at 20,000 lb/in^2. Lysates were centrifuged for 30 min at 39,000 × *g*, then filtered with a 0.45-μm-pore cellulose-acetate filter. The filtrate (soluble fraction) was applied to a preequilibrated Ni-nitrilotriacetic acid (NTA) column (Qiagen, Valencia, CA). The column was washed with 20 bed volumes of buffer A containing 100 mM imidazole, and proteins were eluted with buffer A containing 300 mM imidazole. Control strains BE1355 and BE1356 were grown in parallel with expression strain BE1374, and similar procedures were used for preparing whole-cell extracts and soluble fractions. All protein purification steps were carried out at 4°C, either under strictly anaerobic conditions with deaerated buffers inside a glove box (Coy Laboratory, Grass Lake, MI) and with a nitrogen-hydrogen-CO_2 (90:5:5) atmosphere or under aerobic conditions.

SDS-PAGE and Western blots. Protein concentration was determined using Bio-Rad (Hercules, CA) protein assay reagent with bovine serum albumin (BSA) as a standard. SDS-PAGE was performed using Bio-Rad 12% or 10 to 20% gradient Tris-HCl Ready Gels. Protein bands were visualized by staining with Bio-Safe Coomassie stain (Bio-Rad). For Western blots, the proteins on SDS-PAGE gels were transferred to polyvinylidene difluoride (PVDF) membranes and probed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions, with primary mouse anti-PduS sera at the final concentration of 0.5 μg/ml and secondary goat anti-mouse IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA) at 40 ng/ml.

Gel filtration. Fast protein liquid chromatography (FPLC) was carried out using an ÄKTA system (GE Healthcare, Piscataway, NJ). Aerobically and anaerobically purified PduS-His$_6$ (0.5 ml at a concentration of 20 μM) was applied to a Superdex 200 HR 10/30 column equilibrated with 50 mM sodium phosphate, pH 7.0, and 150 mM NaCl under aerobic conditions. The column was eluted with the same buffer at a flow rate of 0.25 ml/min, and the protein elution profile was determined by monitoring absorbance at 280 nm. Gel filtration standards from Bio-Rad were used to construct a calibration curve of log molecular weight (MW) versus retention time.

Identification of the flavin cofactor bound to PduS-His$_6$. Purified PduS-His$_6$ (in Ni-NTA elution buffer) was boiled for 10 min to release the flavin cofactor. The suspension was rapidly cooled on ice, and denatured protein was removed by centrifugation at 16,000 × *g* for 5 min using an Eppendorf 5415D centrifuge. Flavin-containing supernatant was filtered with a 0.22-μm Millex-GV syringe filter (Millipore, Bedford, MA) and analyzed using a Varian (Palo Alto, CA) ProStar HPLC system, consisting of a model 230 solvent delivery module, a model 430 autosampler, a model 325 UV-visible detector, and a Microsorb-MV 100-5 C_{18} column. The column was developed with a 19-ml linear gradient from 7% to 90% methanol in 5 mM ammonium acetate, pH 6.0, at a flow rate of 1 ml/min. Flavins were detected by monitoring absorbance at 450 nm. Authentic FAD and FMN were used as standards, and flavins separated by high-performance liquid chromatography (HPLC) were collected and analyzed by mass spectrometry (MS).

Cob(III)alamin and cob(II)alamin reduction assays. Enzyme assays were performed under anaerobic conditions as described previously with some modifications (52). All reactions were initiated by addition of NADH or iodoacetate to assay mixtures except where noted in the text. Cob(III)alamin reductase assay mixtures contained 50 mM CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid]-NaOH (pH 9.5), 1.6 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.5 mM NADH, 0.2 mM OH-Cbl, and cob(III)alamin reductase. The conversion of OH-Cbl to cob(II)alamin was monitored spectrophotometrically by measuring the absorbance decrease at 525 nm and quantified using a $\Delta\varepsilon_{525}$ of 4.9 mM^{-1} cm^{-1}, where ε_{525} is the extinction coefficient at 525 nm.

Cob(II)alamin reductase activity was assayed using two methods. In method 1, iodoacetate was used as a chemical trap for cob(I)alamin. Iodoacetate reacts rapidly and quantitatively with cob(I)alamin to form carboxymethylcobalamin (CM-Cbl), with a concomitant increase in absorbance at 525 nm ($\Delta\varepsilon_{525}$ = 5.3 mM^{-1} cm^{-1}) (5). Assay mixtures contained 50 mM CHES-NaOH (pH 9.5), 1.6 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.5 mM NADH, 0.2 mM cob(II)alamin, 0.5 mM iodoacetate, and cob(II)alamin reductase. The second method for measuring cob(II)alamin reduction was a coupled assay with the PduO adenosyltransferase as described in a prior report (52). Assay mixtures contained the same components as those for method 1 except that 0.5 mM iodoacetate was replaced by 0.5 mM ATP and 1.6 μM PduO adenosyltransferase purified from BE118 as described previously (28). PduO converts ATP and cob(I)alamin to AdoCbl; this occurs with an increase in absorbance at 525 nm ($\Delta\varepsilon_{525}$ = 4.8 mM^{-1} cm^{-1}).

Growth studies. Growth studies were performed using a Synergy HT microplate reader (BioTek, Winooski, VT) as previously described (39). For growth of strains carrying plasmids, media were supplemented with 100 μg/ml ampicillin. For complementation studies, IPTG was used at 10 μM to induce expression of genes cloned into pLAC22.

Pdu MCP purification. Cells were grown at 37°C with shaking at 275 rpm to an OD_{600} of 1.0 to 1.2 in 400 ml NCE medium supplemented with 1 mM $MgSO_4$, 0.5% succinate, and 0.6% 1,2-PD and inoculated with 2 ml of an overnight LB culture. Cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. Then, MCPs were purified as previously described and stored at 4°C until used (23).

MALDI-TOF MS-MS. Bands of interest were excised from SDS-PAGE gels and digested "in gel" with trypsin. The resulting peptides were extracted and analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) tandem MS (MS-MS) using a QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an oMALDI ion source. CHCA (α-cyano-4-hydroxy-cinnamic acid) was used as the matrix, and the mass spectrometer was operated in the positive ion mode. Mass spectra for MS analysis were acquired over *m/z* 600 to 2,200. After every regular MS acquisition, two MS-MS acquisitions were performed against the most intensive ions. The molecular ions were selected by an information-dependent acquiring program in the quadrupole analyzer and fragmented in the collision cell. All spectra were processed by a MASCOT (MatrixScience, London, United Kingdom) database search.

Two-hybrid analyses. The *BacterioMatch* II two-hybrid system (Stratagene) was used to detect the potential interactions between the PduS and PduO proteins *in vivo* by following manufacturer's instructions.

RESULTS

Sequence analysis. The PduS protein of *S. enterica* is composed of 451 amino acid residues. Over 100 PduS homologues present in GenBank are associated with 1,2-PD degradation based on gene proximity. PduS also has 43% sequence identity with the RnfC subunit of NADH:ubiquinone oxidoreductase (RnfABCDEG), encoded by the bacterial *rnf* operon (54), and the N terminus of PduS (amino acids 1 to 165) has 25% amino acid identity with the Nqo1 (NDUFV1 in mammals) subunit of the bacterial and mitochondrial NADH-quinone oxidoreductase complex I (49, 53, 57). PduS has several conserved binding sites for substrates and cofactors, including an NADH-binding motif ($^{28}GXGX_2G^{33}$, where X denotes any amino acid) (9), an FMN-binding motif ($^{120}YX_2G[D/E]E^{125}$) (31, 53), and two canonical [4Fe-4S] motifs ($^{264}CX_2CX_2CX_3C^{274}$ and $^{309}CX_2CX_2CX_4C^{320}$) (21, 36) (Fig. 2A and B). PduS also has several conserved domains. An N-terminal glycine-rich loop ($^{25}GX_2GXGGAG[F/L]P[A/T]X_2K^{39}$), proposed to bind the ADP moiety of NADH, is conserved among PduS, RnfC, and Nqo1 (Fig. 2C) (53). A nonclassical Rossmann fold following the Gly-rich loop is proposed to bind FMN (53). A soluble ligand-binding β-grasp fold (SLBB) domain, which belongs to a newly defined superfamily, is proposed to bind cobalamin in PduS (12). The two [4Fe-4S] motifs of PduS are found in a Fer4 domain (PF00037) (12). PduS has a C-terminal sandwich barrel hybrid motif (SBHM). Some SBHM domains carry covalently associ-

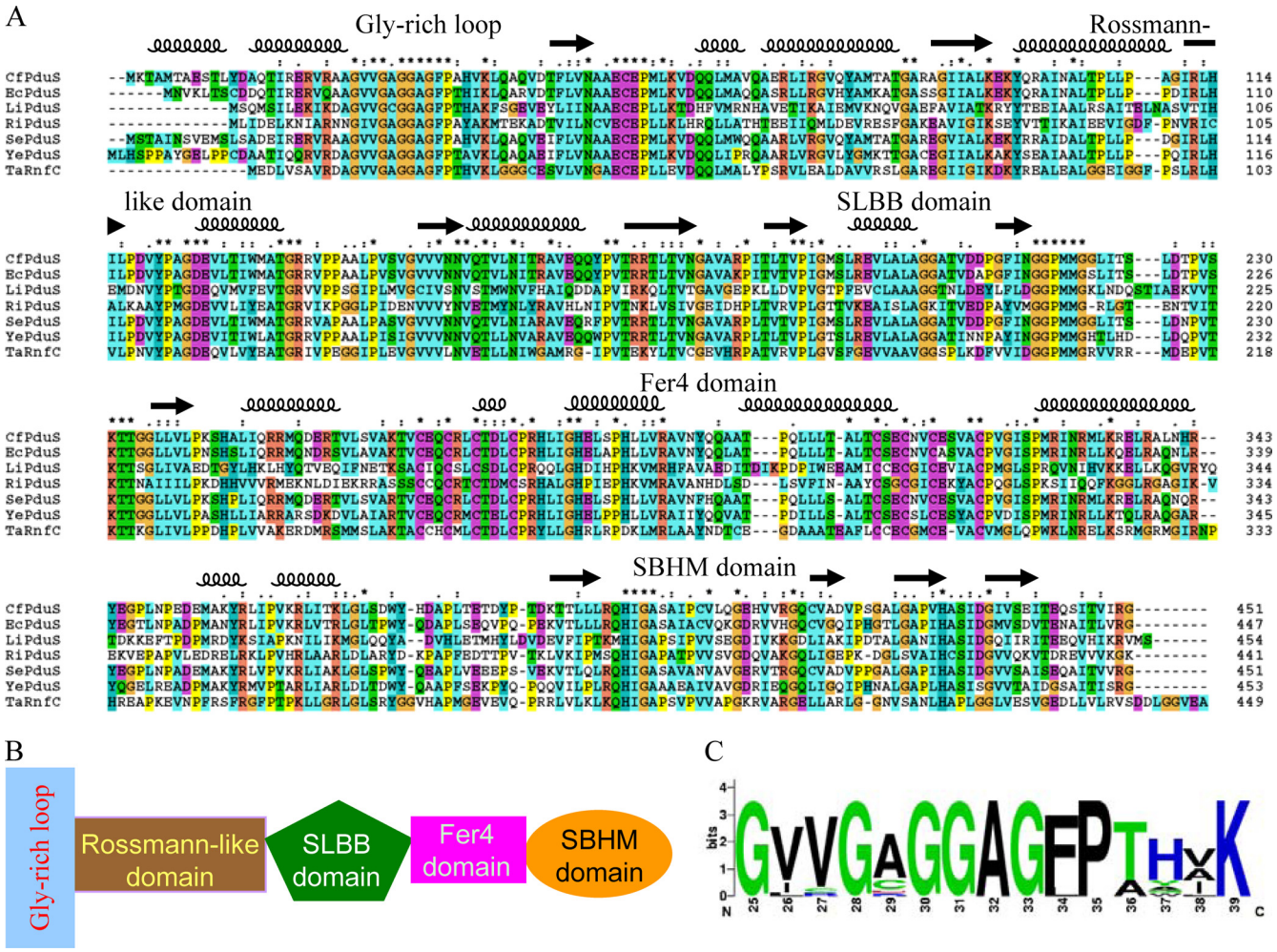

FIG. 2. Sequence analyses of PduS. (A) Structure-based sequence alignment by Clustal X2 and PSIPRED. α-Helix and β-strand are represented by coil and arrow, respectively. CfPduS, PduS from *Citrobacter freundii* (GI 171854200); Ec, *Escherichia coli* (GI 157159374); Li, *Listeria innocua* (GI 16800175); Ri, *Roseburia inulinivorans* (GI 225376079); Se, *Salmonella enterica* (GI 16765383); Ye, *Yersinia enterocolitica* (GI 123442959); TaRnfC, RnfC from *Thermanaerovibrio acidaminovorans* (GI 269791702). (B) The domain architecture of the PduS protein (12). (C) Sequence logo of the amino acid residues (present at positions 25 to 39 in PduS of *S. enterica*) in the glycine-rich region used for nucleotide binding. Polar residues are in green, basic in blue, acidic in red, and hydrophobic in black.

ated ligands, including biotin and lipoate, but PduS has no known covalently bound ligands (12, 26).

Expression and purification of PduS-His$_6$ protein. *E. coli* strain BE1374 was constructed to produce high levels of C-terminal-His$_6$-tagged PduS protein via a T7 expression system. This strain produced relatively large amounts of protein near the expected molecular mass of PduS-His$_6$ (49.2 kDa), and the major portion of this protein was found in the soluble fraction of cell extracts (see Fig. S1 in the supplemental material). In contrast, cell extracts from a control strain containing the expression plasmid without insert (BE1355) produced much less protein near 49.2 kDa. PduS-His$_6$ was purified under both aerobic and anaerobic conditions by Ni-NTA affinity chromatography. Based on SDS-PAGE, anaerobically purified PduS-His$_6$ protein was about 90% homogenous (see Fig. S1, lane 4, in the supplemental material), and a similar level of purity was obtained for aerobically purified enzyme.

Oligomeric state of PduS-His$_6$ protein. Size exclusion chromatography of anaerobically purified PduS-His$_6$ showed a major peak and a small shoulder, which corresponded to a monomer with an apparent molecular mass of 49 kDa and a dimer of 98 kDa, respectively (see Fig. S2A in the supplemental material). Chromatography of aerobically purified PduS-His$_6$ displayed a major peak and a larger shoulder, indicating that the dimer comprised the larger proportion in aerobically purified PduS-His$_6$ (see Fig. S2B in the supplemental material). The small amount of dimerization observed following aerobic purification may have resulted from the formation of intermolecular disulfide bonds, perhaps involving cysteine residues freed from the iron-sulfur clusters, which are oxygen labile.

Characterization of the flavin cofactor of PduS-His$_6$. Aerobically purified PduS-His$_6$ was yellow, and its UV-visible spectrum exhibited peaks at 375 and 450 nm and a shoulder at 480 nm, indicative of a flavin cofactor (see Fig. S3 in the supple-

TABLE 2. Anaerobic purification of PduS-His_6

Purification step	Total protein (mg)	Cob(III)alamin reductase			Cob(II)alamin reductase[a]		
		Sp act (μmol min^{-1} mg^{-1})	Total activity (μmol min^{-1})	Purification (fold)	Sp act (nmol min^{-1} mg^{-1})	Total activity (nmol min^{-1})	Purification (fold)
Whole-cell extract	220	3.9 ± 0.3	858	1	4.4 ± 0.4	968	1
Soluble fraction	166	4.2 ± 0.4	697	1.1	4.9 ± 0.5	813	1.1
Ni-NTA eluate	1.6	42.3 ± 3.2	67.7	10.8	54.5 ± 4.2	87.2	12.4

[a] Iodoacetate at 0.5 mM was used to trap produced cob(I)alamin.

mental material). To determine the binding stoichiometry and the type of flavin (FAD or FMN), purified PduS-His_6 was denatured with heat and the released cofactors were analyzed by HPLC and MS. The flavin extracted from PduS-His_6 eluted at 9.96 min by reverse-phase HPLC, which was similar to the retention time for authentic FMN (9.86 min) (see Fig. S4 in the supplemental material). In contrast, FAD eluted at 8.90 min (see Fig. S4 in the supplemental material). In addition, further studies showed that the flavin released from PduS-His_6 comigrated with authentic FMN by reverse-phase HPLC following coinjection (see Fig. S4 in the supplemental material). Lastly, HPLC and MS were used to confirm and quantify the flavin cofactor of PduS-His_6. The mass spectrum of the flavin released from purified PduS-His_6 showed a major peak with an *m*/*z* of 455.1, corresponding to that of FMN (see Fig. S5 in the supplemental material). Quantitation by HPLC determined a stoichiometry of 0.93:1 (FMN/PduS-His_6), indicating that PduS-His_6 binds 1 molecule of FMN per monomer.

Flavin cofactors may be covalently or noncovalently bound to flavoproteins. The observation that flavin was released from PduS-His_6 by heat treatment indicated noncovalent binding of FMN to PduS-His_6. To further investigate, we used SDS-PAGE. Covalently bound flavins remain protein associated during SDS-PAGE and can be detected by fluorescence following UV illumination of gels (58). In contrast, noncovalently bound flavins are not detected. The FMN cofactor of PduS-His_6 could not be detected by UV illumination of SDS-PAGE gels, indicating noncovalent binding (see Fig. S6 in the supplemental material). In contrast, the flavin of sarcosine oxidase from *Corynebacterium*, which is known to be covalently bound (70), comigrated with the protein during SDS-PAGE and was readily detected by UV illumination (see Fig. S6 in the supplemental material). Thus, results indicate that FMN is noncovalently bound to PduS-His_6.

***In vitro* cob(III)alamin and cob(II)alamin reductase activities of the PduS-His_6 enzyme.** PduS-His_6 was purified using strict anaerobic conditions and tested for cob(III)alamin and cob(II)alamin reductase activities after each purification step (Table 2). The whole-cell extract from the PduS-His_6 expression strain (BE1374) exhibited about 350-fold-higher cob(III)alamin reductase activity (3.9 ± 0.3 μmol min^{-1} mg^{-1}) than did extracts from the control strain carrying the expression plasmid without insert (BE1355) (11.0 ± 3.1 nmol min^{-1} mg^{-1}). Purification of the PduS-His_6 protein by anaerobic Ni-NTA chromatography increased the cob(III)alamin reductase specific activity approximately 11-fold to 42.3 ± 3.2 μmol min^{-1} mg^{-1}.

Assays showed that the PduS-His_6 protein also catalyzed the reduction of cob(II)alamin to cob(I)alamin. Soluble cell extracts of expression strain BE1374 had 4.9 ± 0.5 nmol min^{-1} mg^{-1} cob(II)alamin reductase activity when iodoacetate was used to trap cob(I)alamin and 4.3 ± 0.4 nmol min^{-1} mg^{-1} in a coupled assay with the PduO adenosyltransferase (see assay procedures in Materials and Methods). In contrast, control extracts from BE1355 lacked detectable cob(II)alamin reductase activity. Purification of the PduS-His_6 protein by anaerobic Ni-NTA chromatography increased the cob(II)alamin reductase specific activity about 11-fold to 54.5 ± 4.2 nmol min^{-1} mg^{-1} by the iodoacetate assay and about 9-fold to 40.3 ± 4.1 nmol min^{-1} mg^{-1} by the PduO-linked assay.

PduS-His_6 was also purified under aerobic conditions. Aerobically purified PduS-His_6 retained about 40% activity for both cob(III)alamin and cob(II)alamin reduction. The reduced activity following aerobic purification was likely due to oxidative damage to catalytic sites, such as the iron-sulfur centers predicted from sequence analysis (Fig. 2). The C-terminal His_6 tag had no obvious effect on PduS enzymatic activities based on the comparisons of the reductase activities in the whole-cell extracts and soluble fractions of BE1374 and BE1356, which, respectively, express PduS-His_6 and wild-type PduS.

Reaction requirements. To determine the PduS reaction requirements, key assay components were individually omitted. For cob(III)alamin reduction, there was no activity in the absence of NADH or OH-Cbl. In the absence of PduS-His_6, cob(III)alamin reduction occurred at a rate of 3.5 ± 0.5 nmol min^{-1} (more than 1,100-fold slower than the PduS-catalyzed reaction) due to chemical reduction by NADH. The PduS-His_6 cob(III)alamin reductase lacked measurable activity with CN-Cbl. In the cob(II)alamin reduction assays, controls showed that no detectable CM-Cbl was formed in the absence of NADH, cob(II)alamin, PduS-His_6, or iodoacetate. Similarly, in linked assays with the PduO adenosyltransferase, no AdoCbl was measurable in the absence of NADH, cob(II)alamin, PduS-His_6, ATP, or PduO. Moreover, no cob(I)alamin production could be directly observed by monitoring absorbance at 388 nm (29) in the absence of trapping agents (iodoacetate or PduO and ATP).

Effects of pH, temperature, and divalent metal ions on PduS-His_6 activity. Purified PduS-His_6 showed maximal cob(III)alamin reductase activity at pH 9.5 and 37°C in the presence of Mg^{2+} or Ca^{2+} (see the supplemental material). The cob(II)alamin reductase activity of PduS was not significantly affected by pH (between 7 and 10) and was maximal at 42°C in the presence of Mg^{2+} or Ca^{2+} (see the supplemental material).

Preference for the electron donor of PduS-His_6. To determine the cofactor specificity of PduS-His_6, both cobalamin reductase activities were assayed in the presence of either

TABLE 3. Kinetic parameters for cob(III)alamin and cob(II)alamin reduction by purified PduS-His_6

Reaction	Variable substrate	K_m[a] (μM)	V_{max}[a] (nmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Cob(III)alamin reduction[b]	NADH	10.1 ± 0.7	(43.1 ± 0.5) × 10^3	35.3 ± 0.4	3.5
	OH-Cbl	67.5 ± 8.2	(46.6 ± 1.6) × 10^3	38.2 ± 1.3	0.57
Cob(II)alamin reduction[c]	NADH	27.5 ± 2.4	56.8 ± 1.1	(46.6 ± 0.9) × 10^{-3}	1.7 × 10^{-3}
	Cob(II)alamin	72.4 ± 9.5	64.7 ± 3.6	(53.0 ± 3.0) × 10^{-3}	0.7 × 10^{-3}

[a] The values of K_m and V_{max} were from nonlinear regression using GraphPad Prism 5.
[b] Cob(III)alamin reduction assays were performed with 25 nM anaerobically purified PduS-His_6.
[c] Cob(II)alamin reduction assays were performed with 1 μM anaerobically purified PduS-His_6 protein in the presence of 0.5 mM iodoacetate.

NADH or NADPH at 0.5 mM. Results for purified PduS-His_6 enzyme showed that the relative activity of both cob(II)alamin reductase and cob(III)alamin reductase with NADPH was 16.9% of that with NADH, which was the preferred substrate.

Linearity of the reactions. The effects of PduS-His_6 concentration on enzymatic activities were determined. Cob(III)alamin reduction was proportional to PduS-His_6 concentration from 5 to 50 nM when 0.5 mM NADH and 0.2 mM OH-Cbl were used as substrates. Linear regression yielded an R^2 value of 0.999. Cob(II)alamin reduction, measured in nanomoles of CM-Cbl generated per minute, was linear from 0.2 to 2 μM PduS-His_6 (R^2 = 0.998) when the assay mixture contained 0.5 mM NADH, 0.2 mM cob(II)alamin, and 0.5 mM iodoacetate as the cob(I)alamin trapping agent.

Kinetic analysis of PduS-His_6 activities. Steady-state kinetic studies for cob(III)alamin and cob(II)alamin reduction were performed using purified PduS-His_6 with varied concentrations of one substrate and a fixed concentration of the other substrate. Kinetic parameters were obtained by nonlinear curve fitting, using GraphPad Prism 5 software (GraphPad Software, San Diego, CA), to the Michaelis-Menten equation, $v = V_{max}[S]/(K_m + [S])$, where v is the reaction rate and $[S]$ is the substrate concentration. For cob(III)alamin reduction, the apparent K_m values for NADH and OH-Cbl were 10.1 ± 0.7 μM and 67.5 ± 8.2 μM, respectively (Table 3). The enzyme V_{max} values were 43.1 ± 0.5 and 46.6 ± 1.6 μmol min^{-1} mg^{-1} when OH-Cbl and NADH, respectively, were held at constant levels. The fixed concentrations of OH-Cbl and NADH used were 200 μM and 500 μM, respectively. Five hundred micromolar NADH is 50-fold higher than the apparent K_m (98% saturating), and 200 μM OH-Cbl is 3-fold higher than the apparent K_m (75% saturating); the latter was the highest level that could be used while still retaining a linear response from the spectrophotometer.

The kinetics of cob(II)alamin reduction were determined using iodoacetate to trap cob(I)alamin. Nonlinear regression indicated apparent K_ms of 27.5 ± 2.4 μM and 72.4 ± 9.5 μM for NADH and cob(II)alamin, respectively (Table 3). The enzyme V_{max}s were 56.8 ± 1.1 nmol min^{-1} mg^{-1} when the cob(II)alamin concentration was held constant and 64.7 ± 3.6 nmol min^{-1} mg^{-1} when NADH was held at a saturating level. For the above kinetic studies, NADH and cob(II)alamin were used at fixed concentrations of 500 μM and 200 μM, respectively.

Phenotype and complementation of a *pduS* deletion mutation. To investigate the function of PduS *in vivo*, we measured the growth of a *pduS* deletion mutant (BE1352) on 1,2-PD minimal medium. BE1352 grew slowly (64% of the wild-type rate) on 1,2-PD minimal medium supplemented with a saturating level of CN-Cbl (100 nM) (Fig. 3A). At limiting concentrations of CN-Cbl (20 nM), the *pduS* deletion mutant grew more slowly (61%) and to a lower cell density than wild-type *S.*

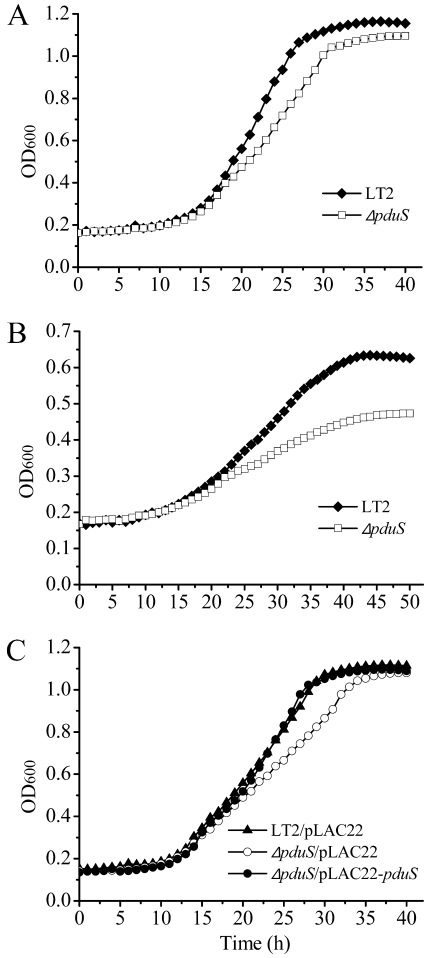

FIG. 3. Phenotype and complementation of a *pduS* deletion mutation. Cells were grown in NCE minimal medium with 1,2-PD as the sole carbon and energy source. (A) The *pduS* deletion moderately impaired growth on 1,2-PD supplemented with 100 nM CN-Cbl. (B) The *pduS* deletion impaired growth and decreased cell density on 1,2-PD with limiting CN-Cbl (20 nM). (C) The *pduS* deletion was complemented by ectopic expression of *pduS* at 100 nM CN-Cbl. IPTG at 10 μM was used to induce production of PduS from pLAC22.

TABLE 4. Two-hybrid analysis of PduS-PduO interactions

Plasmid pair	No. of colonies[a]			
	Nonselective medium		Selective medium (3-AT)	
	1/100	1/1,000	1/10	1/1,000
pBT-LGF2/pTRG-Gal11[b]	TNTC[c]	~2,000	TNTC	~2,000
pBT-*pduS*/pTRG-*pduO*	>2,000	255	>2,000	152
pBT-*pduO*/pTRG-*pduS*	>2,000	296	>2,000	129
pBT-*pduS*/pTRG	>2,000	410	0	0
pBT/pTRG-*pduS*	>2,000	564	1	0
pBT-*pduO*/pTRG	>2,000	170	2	0
pBT/pTRG-*pduO*	>2,000	400	3	0

[a] Number of colonies formed following cotransformation of the reporter strain with the bait and target plasmids on selective and nonselective media at the dilution indicated.
[b] LGF2 and Gal11 are positive controls known to strongly interact.
[c] TNTC, too numerous to count.

enterica (Fig. 3B). Complementation analysis was also conducted. Production of PduS from pLAC22 fully corrected the slow-growth phenotype of the Δ*pduS* mutant at saturating CN-Cbl (Fig. 3C) and limiting CN-Cbl (not shown), confirming that slow growth was due to the *pduS* deletion and not due to polarity or an unknown mutation. The growth curves shown in Fig. 3A to C were all repeated at least three times with triplicate cultures. The slow-growth phenotype of the *pduS* deletion mutant supports a role for PduS in cobalamin reduction *in vivo* as further described in Discussion.

Interaction between PduS and PduO. Prior studies suggested the PduS and PduO interact *in vitro*, which makes sense because they catalyze sequential reactions in cobalamin recycling (52). To examine the potential interactions between PduS and PduO *in vivo*, two-hybrid analyses were performed. For the system used, detection of protein-protein interactions is based on transcriptional activation of the *Saccharomyces cerevisiae HIS3* reporter gene, which confers 3-amino-1,2,4-triazole (3-AT) resistance. In application, a reporter strain is cotransduced with a target and bait plasmid pair and the number of 3-AT-resistant colonies is used to estimate the strength of the protein-protein interaction. To test for a PduS-PduO interaction, the colonies on nonselective and selective (3-AT) media were counted at different dilutions after cotransformation (Table 4). When PduO and PduS were produced from the target (pTRG) and bait (pBT) plasmids or vice versa, a substantial number of 3-AT-resistant cotransformants were obtained. In contrast, very few 3-AT-resistant transformants were observed in controls that lacked either *pduO* or *pduS* (Table 4). These results indicated that PduS interacts with PduO *in vivo*.

PduS is a component of Pdu MCP. To determine the cellular location of PduS, MCPs purified from wild-type *S. enterica* and *pduS* deletion mutant BE1352 were analyzed by SDS-PAGE, MALDI-TOF MS-MS, and Western blotting. A band near the expected molecular mass of PduS (48.4 kDa) and close to the PduP band at 49.0 kDa was observed on an SDS-PAGE gel of MCPs purified from the wild-type strain (Fig. 4A lane 2). This band was further analyzed by MALDI-TOF MS-MS. Five sequences (KSHPLIQRR, RAIDALTPLLPDGIRL, KVDQQL MWQQAARL, RQHIGASAVANVAVGERV, and RHLIG HELSPHLLVRA) identified by MS-MS of a trypsin digest matched the PduS protein, indicating that it is a component of purified Pdu MCPs. In addition, Western blotting with PduS antisera detected a band near 48 kDa in MCPs purified from wild-type *S. enterica* (Fig. 4B, lane 2), while no PduS band was detected in MCPs purified from the *pduS* deletion mutant (Fig. 4B, lane 4). Furthermore, when similar amounts of protein were analyzed, Western blotting easily detected PduS in purified MCPs but not in crude cell extracts (Fig. 4B, lanes 2 and 1). This indicated that purified MCPs were substantially enriched in PduS. Cumulatively, these results show that PduS protein is a component of Pdu MCPs.

DISCUSSION

In previous studies, an *E. coli* strain that produced high levels of the PduS enzyme of *S. enterica* was constructed (52). Crude cell extracts from this strain had substantially increased cob(III)alamin and cob(II)alamin reductase activities, suggesting that the PduS enzyme played a role in cobalamin assimilation and recycling (52). Very recently, the PduS enzyme was purified and shown to be a flavoprotein that can supply electrons for the synthesis of AdoCbl by the human and *Lactobacillus* ATR enzymes; however, PduS was not further characterized (40). In this report, C-terminally His_6-tagged PduS protein was purified and characterized more extensively. Results showed that PduS is a flavoprotein that contains 1 FMN per monomer. Anaerobically purified PduS-His_6 protein exhibited cob(III)alamin and cob(II)alamin reductase activities of 42.3 μmol min^{-1} mg^{-1} and 54.5 nmol min^{-1} mg^{-1}, respectively. Although, the cob(II)alamin reductase activity is 776-fold lower than the cob(III)alamin reductase activity, it is reasonable to infer that this activity is relevant *in vivo*. The midpoint potentials of cob(III)alamin/cob(II)alamin (+240 mV) and cob(II)alamin/cob(I)alamin (−610 mV) (1, 37) rationally account for the incongruity between the two activities. AdoCbl is needed only in very small quantities, generally from 1 to 10 μM in prokaryotes (56). At least one other enzyme with a well-documented role in AdoCbl synthesis (the CobA ATR)

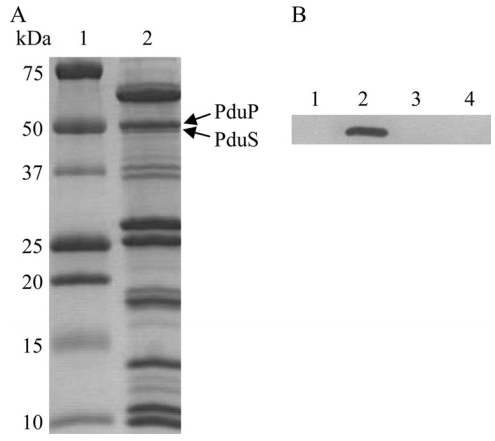

FIG. 4. PduS is a component of the Pdu MCP. (A) Ten to 20% SDS-PAGE gel stained with Coomassie brilliant blue. Lane 1, molecular mass markers; lane 2, 10 μg Pdu MCPs purified from wild-type *Salmonella*. (B) Western blot for PduS. Lanes 1 and 2, 10 μg whole-cell extract or purified MCPs from the wild type; lanes 3 and 4, 10 μg whole-cell extract or purified MCPs from BE1352 (Δ*pduS*).

was reported to have a specific activity of 53 nmol min^{-1} mg^{-1} (64), which is very close to the cob(II)alamin reductase activity of PduS reported here. Moreover, the disparity in cob(III)alamin and cob(II)alamin reductase activities of PduS is similar to that reported for the CobR corrin reductase from *Brucella melitensis*, which is unrelated in amino acid sequence to PduS but catalyzes similar reactions (32). Thus, we infer that the lower cob(II)alamin reductase activity of PduS is within a physiologically relevant range.

Kinetic studies of purified PduS-His_6 were also performed. The apparent K_m values of PduS-His_6 were 67.5 μM and 10.1 μM for OH-Cbl and NADH, respectively, in cob(III)alamin reduction and 72.4 μM for cob(II)alamin and 27.5 μM for NADH in cob(III)alamin reduction. Typical intracellular cobalamin levels are about 1 to 10 μM in prokaryotic organisms (56), and the levels of nicotinamide coenzymes are 1 to 3 mM (45). This suggests that *in vivo* NADH would be saturating and cobalamin would be limiting for PduS activity; however, results presented in this report indicate that PduS is a component of the Pdu MCP and hence may reside in the MCP lumen, where the local cobalamin concentration is unknown but might be elevated relative to that in the cytoplasm of the cell. Thus, the K_m values of PduS for cobalamins might reflect its localization within the lumen of the Pdu MCP.

Several other systems capable of reducing cobalamin *in vitro* have been previously described (18, 19, 32, 40, 52). The reduction of cob(III)alamin to cob(II)alamin is facile and certainly occurs to some extent in the cytoplasm of prokaryotic cells (which is a strongly reducing environment) without the requirement for a specific reductase (19). In addition, several flavoproteins as well as reduced FMN ($FMNH_2$) and reduced FAD ($FADH_2$) reduce cob(III)alamin to cob(II)alamin *in vitro* and may contribute to cob(III)alamin reduction *in vivo* (19, 40, 52). The reduction of cob(II)alamin to cob(I)alamin is more difficult due to the extremely low redox potential of the cob(II)alamin/cob(I)alamin couple (−610 mV) (37). Prior studies showed that $FADH_2$, $FMNH_2$, ferredoxin $NADP^+$ reductase (Fpr), flavodoxin A (FldA), and flavin:NADH reductase (Fre), as well as flavoproteins with no known involvement in B_{12} metabolism, can provide electrons for cob(II)alamin reduction *in vitro* in the presence of ATR (19, 40). Results indicated that these systems specifically reduce 4-coordinate ATR-bound cob(II)alamin, whose midpoint potential is about 250 mV higher than that of 5-coordinate cob(II)alamin (40, 60). In this report, we showed that purified PduS is able to reduce both cob(III)alamin and cob(II)alamin in the absence of additional proteins. The reduction of cob(II)alamin to cob(I)alamin was detected by trapping cob(I)alamin with iodoacetate and by using a coupled assay with the PduO ATR. In both assays, the rates of cob(II)alamin reduction were similar: 54.5 and 40.3 nmol min^{-1} mg^{-1}, respectively. This showed that ATR was nonessential for cob(II)alamin reduction by PduS. This raises the question of how PduS overcomes the redox potential barrier for cob(II)alamin reduction. One possibility is that PduS binds cob(II)alamin and converts it to the 4-coordinate form prior to reduction.

The fact that cobalamin can be reduced by $FMNH_2$, $FADH_2$, and various flavoproteins raises the question of the relative contributions of these systems *in vivo*. To examine the role of PduS *in vivo*, we tested the effects of a *pduS* deletion mutation on AdoCbl-dependent growth on 1,2-PD with saturating or limiting CN-Cbl (Fig. 3A and B). Under both conditions, the *pduS* deletion impaired growth, indicating that PduS was required to support the maximal rate of 1,2-PD degradation under the conditions used. The likely reason that the *pduS* deletion produced only a partial growth defect is that *S. enterica* has multiple cobalamin-reducing systems, as described above. A similar partial growth defect on 1,2-PD was observed following genetic deletion of the *Salmonella* PduO ATR, which is partially redundant with the CobA enzyme (29). Thus, results presented here indicate that PduS provides additional cobalamin reduction capacity required for maximal growth on 1,2-PD and may also be important within the confines of the Pdu MCP as discussed below.

Results presented here demonstrated that PduS is associated with Pdu MCPs. PduS was identified as a component of purified MCPs following SDS-PAGE (Fig. 4A) and MS-MS. In addition, Western blotting showed that purified MCPs were substantially enriched in PduS, which established a specific association between PduS and the Pdu MCP (Fig. 4B). Furthermore, two-hybrid studies indicated an *in vivo* interaction between PduS and PduO, which was previously localized to the Pdu MCP (23). Prior proteomics studies missed PduS as an MCP component (23). This was likely due to the difficulty in separating PduS from the PduP enzyme (a major MCP component) by SDS-PAGE (Fig. 4A). Alternatively, PduS may have been missed due to its high pI (8.82), which would have resulted in migration to the edge of the gel or off it during the isoelectric focusing step of two-dimensional (2D) electrophoresis used in prior studies (23). Thus, substantial evidence indicates PduS is a component of the Pdu MCP. This establishes that all the enzymes needed for cobalamin recycling are components of the Pdu MCP, suggesting that cobalamin recycling can occur entirely within the lumen of the Pdu MCP (Fig. 5). The AdoCbl-dependent diol dehydratase (PduCDE) required for 1,2-PD degradation, which has been localized to the lumen of the Pdu MCP, is subject to mechanism-based inactivation and requires cobalamin recycling for activity (43, 44). This significantly revises our understanding of how the Pdu MCP functions.

The finding that PduS is involved in cobalamin recycling might provide an insight into cobalamin reduction in human mitochondria, where the reductive reactions needed for the synthesis of AdoCbl are not well understood. AdoCbl is an essential cofactor for mitochondrial methylmalonyl-CoA mutase (MUT or MCM), which converts methylmalonyl-CoA to succinyl-CoA during the catabolism of branched-chain amino acids, thymine, uracil, cholesterol, and odd-chain fatty acids (17). The current view is that cobalamin, probably as cob(II)alamin, is transported into the mitochondria, where the second reduction, yielding cob(I)alamin, occurs, followed by adenosylation by human ATR to form AdoCbl (3). However, the human mitochondrial cobalamin reductase(s) has not been identified. An earlier study showed that human methionine synthase reductase (hMSR) can act as the reducing enzyme in combination with human ATR (hATR) to generate AdoCbl from cob(II)alamin *in vitro* (34). Initially, this suggested that hMSR might function as the elusive cobalamin reductase in mitochondria, but this possibility now seems unlikely since a recent report indicates that the hMSR protein is

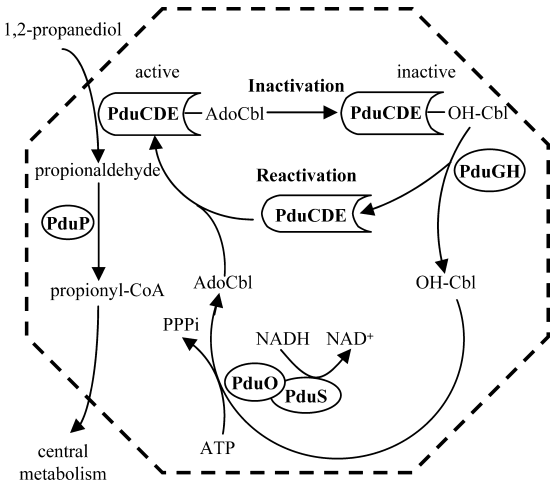

FIG. 5. Proposed model for cobalamin recycling inside the Pdu MCPs of *S. enterica*. Prior studies showed that AdoCbl-dependent diol dehydratase (PduCDE) resides in the lumen of the Pdu MCP. PduCDE is subject to mechanism-based inactivation in which a damaged Cbl cofactor (OH-Cbl) becomes tightly enzyme bound. Diol dehydratase reactivase (PduGH) releases OH-Cbl from PduCDE. Then, OH-Cbl is reduced by PduS to cob(I)alamin and adenosylated to AdoCbl by the PduO adenosyltransferase. AdoCbl spontaneously associates with apo-PduCDE to form active holoenzyme. The recycling of OH-Cbl is required to maintain the activity of PduCDE and hence is essential for the degradation of 1,2-propanediol as a carbon and energy source. The finding that PduS is a component of the Pdu MCP suggests that cobalamin recycling can occur entirely within the MCP lumen.

restricted to the cytosol (20). Our sequence analyses showed that the Nqo1 subunits of bovine and human mitochondrial respiratory complex I have 25% amino acid identity (44% similarity; expect = 8×10^{-9}) to the N-terminal region (amino acids 1 to 165) of the PduS protein of *S. enterica*. PduS and Nqo1 also share SLBB domains (amino acids 173 to 221 in PduS), which have 33% amino acid identity (expect = 7×10^{-4}), and this domain was proposed to bind cobalamin in the PduS protein (12). Moreover, PduS and Nqo1 both contain binding sites for NADH, FMN, and an iron-sulfur cluster involved in electron transfer (Fig. 2B). Although there is currently no evidence for a soluble ligand interacting with the SLBB domain in Nqo1, it's possible that cobalamin binds this domain and accepts electrons from Nqo1 given the similarity between PduS and Nqo1. Hence, Nqo1 might have the dual functions of electron transport for a respiratory chain and cobalamin reduction for AdoCbl synthesis, although this is speculative at this time.

ACKNOWLEDGMENTS

This work was supported by NSF grant MCB0956451 to T.A.B.

We are indebted to Yasuhiro Takahashi from the Division of Life Science at Saitama University (Japan) for offering plasmids pRKNMC, pRKISC, and pRKSUF017. We thank Siquan Luo from the Proteomics Facility at Iowa State University for aid with MS-MS analyses, Tracie A. Bierwagen in the ISU BBMB Department for help with gel filtration, the ISU DNA Sequencing and Synthesis Facility for assistance with DNA analyses, and the ISU W. M. Keck Metabolomics Research Laboratory for assistance with the mass spectrometry analyses.

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